Biochemical Pharmacology, Vol. 52, pp. 15–19, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00109-8

Ebselen-Binding Equilibria Between Plasma and Target Proteins

Volker Ullrich, Peter Weber, Frank Meisch and Frank von Appen*
DEPARTMENT OF BIOLOGY, UNIVERSITY OF KONSTANZ, BOX 5560 M611, D 78434 KONSTANZ, GERMANY

ABSTRACT. The antiinflammatory drug ebselen (2-phenyl-1,2-benzisoselenazo-3(2H)-one) is known to bind covalently to thiols to form seleno disulfides that, directly or indirectly, are responsible for its pharmacological effects. Due to its reactive thiol group and high plasma concentration, albumin is a preferred target of ebselen, which it binds covalently. Ebselen should not, then, be available for intracellular actions at other target proteins. We have addressed this question, and show by difference spectroscopy that the interaction of ebselen with albumin occurs stoichiometrically under ring opening, but is readily reversible in the presence of glutathione. With intact human polymorphonuclear leukocytes (PMN), a similar stoichiometric reaction with distinct spectral features was observed with ebselen that was completely abolished by pretreatment of PMN with N-ethylmaleimide, but not by selective depletion of cellular glutathione. Human platelets, again, exhibited different spectral changes upon addition of ebselen. In agreement with results reported in the literature, we show that ^{14}C-ebselen is in dynamic equilibrium with all accessible thiol groups and, hence, despite mostly being bound covalently to albumin, it will exchange rapidly with other target proteins in PMN or platelets. BIOCHEM PHARMACOL 52;1:15–19, 1996.

KEY WORDS. thiol group; albumin; polymorphonuclear leukocytes; platelets; difference spectroscopy; covalent binding; ebselen

Ebselen has been developed as a drug with antiinflammatory actions that have been related to a surprisingly high number of physiological targets in the organism. Its main function may be that of a glutathione peroxidase mimic [1–3], but many other pharmacological and biological effects have also been reported [4–7]. Invariably, the selenium atom, as a center for nucleophilic attack and of unusual redox chemistry, has been suggested to be involved in all reactions. On the other hand, it was puzzling to observe such activities because intracellularly GSH,† as a strong nucleophile, will react rapidly and quantitatively with ebselen [8] and will be the preferred target in plasma albumin, leading to a tight binding of ebselen to the thiol groups of albumin [9, 10] For its intracellular action, the problem was solved by the finding that the primary reaction product, ebselen selenodisulfide, can be reduced by a second GSH molecule to yield selenol. By analogy to the enzymatic mechanism of GSH peroxidase, selenol is an excellent reductant for peroxides and the selenic acid product can be cycled back to the selenodisulfide through the action of GSH [1]. This establishes an efficient peroxidase-mimetic cycle within the cells. Less clear was the pathway by which ebselen entered the cells from the blood. Therapeutically active concentrations require ebselen blood levels above 2 mM, of which more than 90% is bound to albumin. In a recent study, Wagner *et al.* [10] presented evidence that ^{14}C-ebselen from the albumin-adduct was transferred to intracellular proteins of hepatocytes. An active role of membrane-bound thiol groups was proposed because the transfer through a dialysis membrane required the presence of reductants like GSH or mercaptoethanol. Because PMN are the most likely targets for the antiinflammatory action of ebselen and the chemical nature of the adducts was not yet clarified, we concentrated on these aspects of the molecular pharmacology of ebselen.

MATERIALS AND METHODS

Materials

Ebselen was kindly provided by Rhone-Poulenc-Rorer (Paris, France) and ^{14}C-ebselen (1850 MBg/mmol) was synthesized according to the literature with ^{14}C-labeled 2-methylseleno benzoic acid as the starting material, for which we are grateful to Dr. N. Dereu (Rhone-Poulenc-Rorer, Paris, France) [11]. Albumin (delipidated), N-ethylmaleimide, dinitrochlorobenzene, and glutathione were obtained commercially in the highest purity available.

* Corresponding author: Prof. Volker Ullrich, Universität Konstanz, Fakultät für Biologie, Postfach 5560 M611, D 78434, Konstanz, Germany. Tel. (49) 7531 882287; FAX (49) 7531 883099.

† *Abbreviations:* GSH, reduced glutathione; NEM, N-ethylmaleimide; PMN, polymorphonuclear leukocytes; BSA, bovine serum albumin. Enzymes: glutathione peroxidase, EC 1.11.1.9; glutathione-S-transferase, EC 2.5.1.18.

Received 20 September 1995; accepted 2 February 1996.

Blood Fractions

Blood (200 mL) from healthy volunteers was collected in 0.38% citrate solution and platelet-rich plasma was prepared as described [12]. Washed platelets [13] and PMN [14] were isolated as previously described.

Difference Spectroscopy

Difference spectra were recorded in 10-mm tandem cuvettes of 1 mL volume by means of an Aminco-Chance DW-2 double beam spectrometer. A single recording required no more than 2 min. The spectra were digitized and processed by a home-built PC-based device. A stock solution of 10 mM ebselen in ethanol was used.

Equilibrium Dialysis

Dialysis was carried out at room temperature in a polyethylene block with 5 double chambers of 500 μL each, with a separating Visking Type 20/32 membrane (Serva, Heidelberg/New York). Each cell could be loaded by a syringe and the content was withdrawn after the end of the experiments for radioactivity counting in a PW4700 Philips β-counter.

Purification of Ebselen-Bound Albumin

Albumin (50 mg) was incubated in 1.5 mL of water with 1.6 μmol of [^{14}C]-ebselen [3 MBq] dissolved in 50 μL of ethanol. After stirring for 30 min, the mixture was chromatographed on a Sephadex G10 column and the albumin fraction collected. It contained 0.61 mol ebselen/mol albumin and was stored at −70°C.

RESULTS

Ebselen exhibits an absorption band in the uv at 346 nm, which permits determination of its spectral interaction with albumin (Fig. 1). In this difference spectrum, a decrease at 346 nm occurred concomitantly with the formation of a band at 297 nm and a shoulder at 257 nm. The titration curve showed saturation at 77 μM and, because the albumin concentration was 150 μM, about half of the albumin must have reacted. Albumin in the presence of NEM did not give any spectral change, indicating that the SH-group of albumin was involved and that, in our preparation, about half of these thiol groups were already modified.

A comparison of the difference spectra of ebselen with GSH qualitatively confirms the blueshift upon reaction with a thiol group but also indicates that, with albumin, an additional decrease at 275 nm occurs that is in the range of the aromatic aminoacids. This may indicate that ebselen also reacts with other protein groups. The lack of an isosbestic point supports the assumption of additional reactions, but it should also be kept in mind that a slow conversion to ebselen diselenide will change the difference spectra further [15].

An important aspect in the aim of this study was the

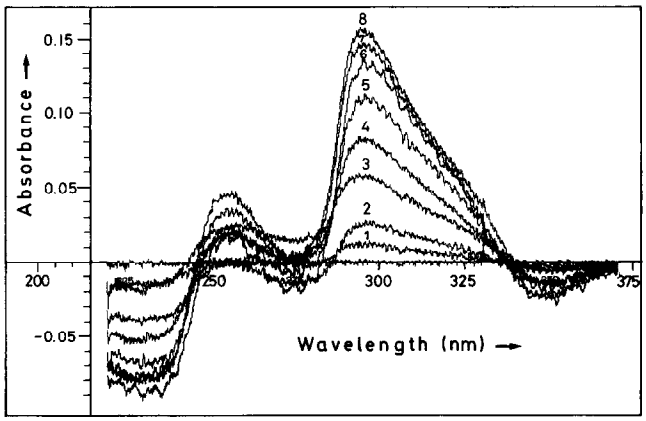


FIG. 1. Difference spectra of bovine serum albumin with ebselen. To 10 mg albumin/mL in 0.1 M Tris HCl pH 7.4, increasing concentrations of ebselen (1–5 = 10–50 μM; 6 = 75 μM; 7 = 100 μM; 8 = 150 μM) were added with corresponding amounts of ethanol to the albumin compartment of the reference cuvette; ebselen was added to the second compartment of the reference cuvette. A typical experiment out of three. Additions were made in 2-min time intervals.

affinities of ebselen towards GSH and protein thiol groups when present together. With 10^{-4} M GSH and 0.75×10^{-4} M albumin, the difference spectrum in Fig. 2 was observed, indicating that the affinity of ebselen to GSH was higher than that to albumin (results not shown). Human plasma at a 1:10 dilution upon titration with ebselen qualitatively gave the same spectral change up to about 10^{-4} M ebselen (results not shown).

Because we intended to study ebselen binding in a plasma-PMN system, we also determined the spectral changes when ebselen was incubated with isolated human PMN (Fig. 3). Although qualitatively similar to the ebselen difference spectra with GSH and albumin, two differences can be observed. First, the isosbestic point shifted from 319

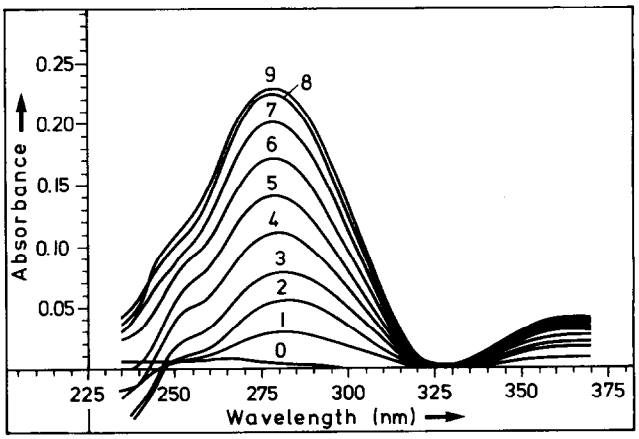


FIG. 2. Spectral interaction of ebselen with glutathione. To a concentration of 100 μM GSH, increasing amounts (1–5 = 10–50 μM; 6 = 15 μM; 7 = 100 μM; 8 = 200 μM; 9 = 300 μM) of ebselen were added, with corresponding amounts of ethanol and ebselen added to the reference tandem cuvette. Additions were made in 2-min time intervals.

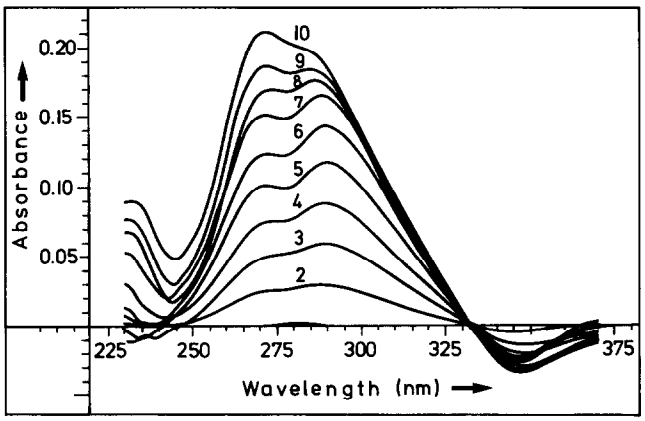


FIG. 3. Ebselen-induced difference spectra with human PMN. To a suspension of 10^6 PMN/mL in PBS-buffer, 20–100 µM of ebselen (in ethanol) was added and the difference spectra recorded immediately (<1 min).

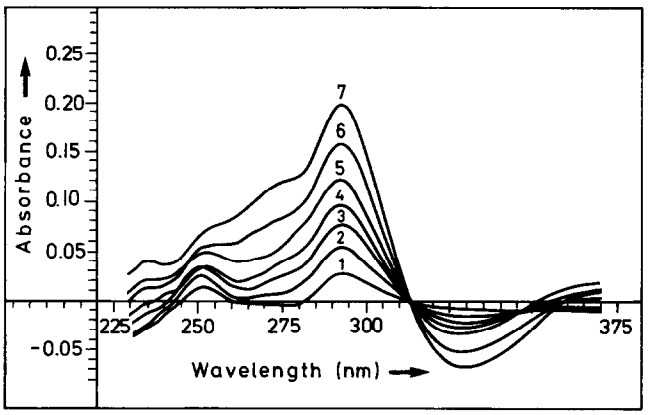


FIG. 5. Difference spectra of ebselen with human platelets. Washed human platelets (OD 1–1.5) were suspended in PBS buffer and 10–50 µM (curve 1–5), 75 µM (curve 6), and 100 µM ebselen were added.

to 334 nm, and the trough at 275 nm was much less pronounced, as with albumin, but definitely not absent as observed with GSH alone. As a control, PMN were treated with NEM under conditions that allowed a complete alkylation of the thiol groups of GSH and proteins (Fig. 4). As expected, no difference spectrum remained. However, if PMN were treated with chlorodinitrobenzene to deplete only intracellular GSH by the action of GSH transferase, a difference spectrum with an isosbestic point at 334 nm and a band at 295 nm was still formed. In a control experiment with the same concentrations, no spectral change was observed with CDNB and ebselen alone.

Because human platelets are also targets of ebselen in plasma, we applied the difference spectroscopy to ebselen and washed human platelets (Fig. 5).

Showing a clear isosbestic point at 313 nm, an absorption band appeared at 294 nm with a very pronounced trough at around 328 nm. This trough had the shape of the absorption band of ebselen, which would indicate that ebselen was forming a compound with platelet constituents that absorbs at very different wavelengths from those of ebselen and has no significant absorption around 300 nm.

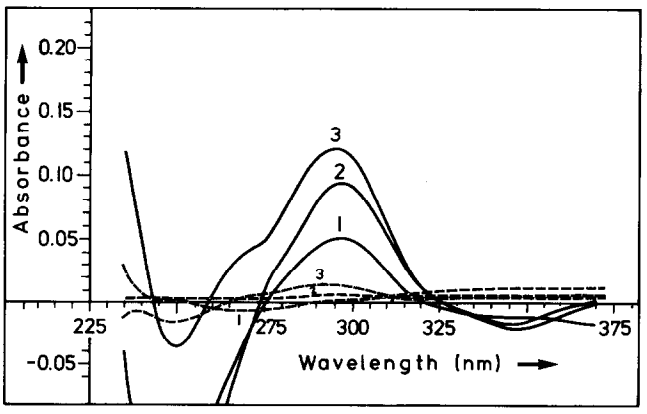


FIG. 4. Effect of SH reagents on ebselen-induced difference spectra in human PMN. PMN (10^6/mL) were pretreated with NEM (1 mM) and CDNB (10 µM solid lines) for 1 hr and the difference spectra with ebselen of curves 1 (50 µM), 2 (100 µM), and 3 (200 µM) were recorded.

Binding and Equilibria Studies

For these studies, ^{14}C-labeled ebselen was synthesized as described in the literature [11]. In a first experiment, ^{14}C-ebselen was dialyzed against albumin and the curves depicted in Fig. 6 were obtained.

In the initial first-order reaction with $\tau_{1/2}$ = 1.5 hr, binding of ebselen to albumin occurred until about 30% of the

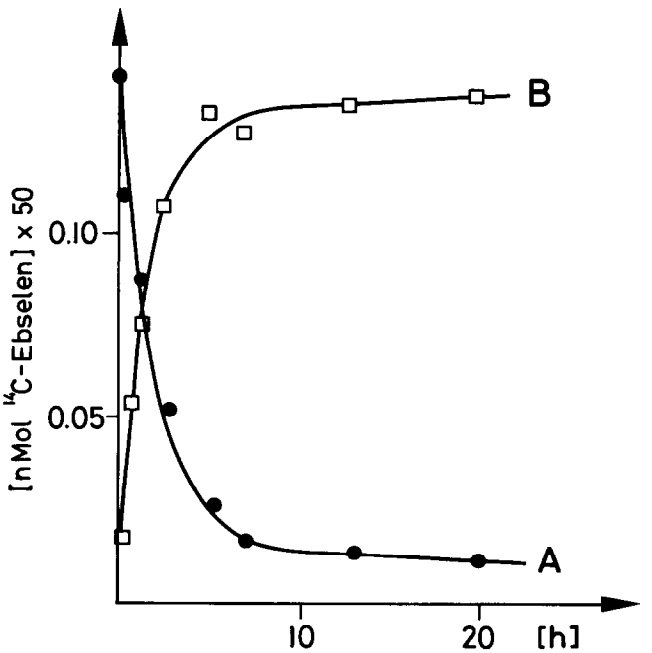


FIG. 6. Dialysis of ^{14}C-ebselen against bovine serum albumin. In chamber A, the decreasing concentration of added ^{14}C-ebselen with time was monitored by a β-counter with corresponding measurements in chamber B.

albumin was occupied on a molar basis. At least part of the label was covalently bound because, on a non-reducing gel, the radioactivity present in chamber B could be found at 69 kD (result not shown).

If this albumin fraction was passed over a G25 Sephadex column and then dialyzed against the same concentration of albumin, a slow exchange of [^{14}C] ebselen was observed; however, this was greatly increased in the presence of 0.5 mM glutathione (Fig. 7). This effect on GSH was concentration-dependent.

Because this finding is in agreement with other reports in the literature [10], we did not extend these experiments further with regard to GSH concentrations and other thiol reductants. It was interesting to note, however, that in contrast to these reports, we observed dialysis even in the absence of any low-molecular-weight thiols and that this transfer continued with about the same kinetics after GSH had acted. As a simpler explanation, some portion of ^{14}C-ebselen may have been bound noncovalently, with its exchange being unaffected by GSH addition.

When albumin-bound ebselen, in the absence of GSH, was dialyzed against human PMN for 12 hr, only negligible amounts (less than 5%) were found in the PMN. Addition of GSH increased this amount severalfold in agreement with the previous results. Assuming an average volume per PMN of 3×10^{-13} L, then 10^7 PMN cells represent an intracellular volume of 3×10^{-6} L. From the amount of ^{14}C-ebselen bound to the PMN, this would correspond to a concentration of 43 μM whereas, in the outside medium, about 1.8 μM were measured. Hence, an enrichment of more than 20-fold had taken place. In a final experiment, whole blood was used, to which ^{14}C-ebselen was added, and its concentration in PMN was measured. Using 13.5 mL of freshly drawn human blood and 5238 nmol of ^{14}C-ebselen (27×10^6 dpm), the PMN fraction was isolated and counted for radioactivity. The serum fraction was applied to SDS-gel electrophoresis. Using a volume of 3×10^{-13} L per PMN and a yield of 1.9×10^7 PMN a volume of 5.7×10^{-6} L of PMN was obtained, for which a cellular concentration of 5.1×10^{-4} M ebselen was calculated. Because the overall concentration of ebselen in whole blood was 3.88×10^{-4} M, it is obvious that the plasma plus erythrocyte binding competes well with that of PMN, which is understandable when one calculates that 11.7 μmol of albumin were present compared to the 5.2 μmol ebselen employed. After SDS-electrophoresis, radioactivity comigrated with albumin (results not shown).

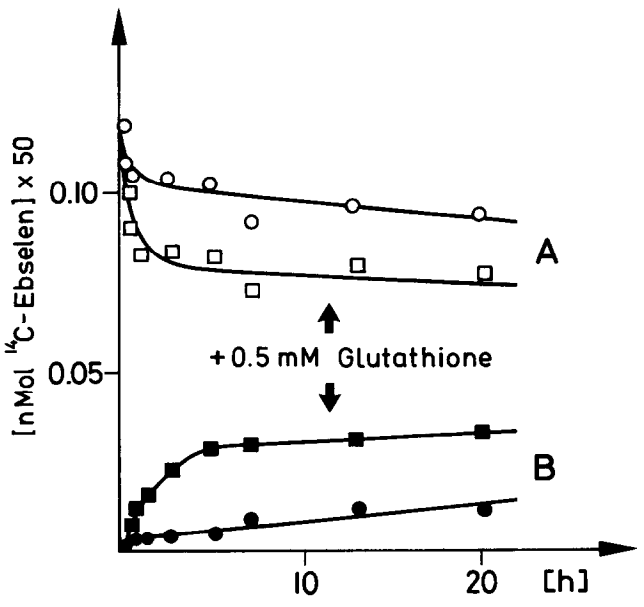


FIG. 7. Dialysis of ^{14}C-albumin-bound ebselen against PBS-buffer with and without glutathione. ^{14}C-ebselen was bound to BSA and purified as described in Methods. Dialysis was carried out over the time indicated at room temperature in the absence and presence of 0.5 mM glutathione.

DISCUSSION

Our result, of albumin binding of ebselen, confirmed published data and can explain the unusual pharmacokinetic behavior of this antiinflammatory drug. We verified that the binding must be covalent, at least in part, because the ^{14}C-labeled drug would otherwise not have been migrated with the protein on SDS-gel. Glutathione is able to catalyze an exchange of this covalently bound ebselen, which is consistent with the formation of a mixed selenodisulfide at the thiol group of albumin and its transthiolation to GSH, forming the ebselen selenodisulfide with glutathione. Also, the spectral data support an opening of the heterocyclic ring of ebselen with its absorption band around 346 nm and the formation of benzanilide derivatives absorbing between 270 and 300 nm, depending on the thiol compound. Characteristic of the albumin-ebselen difference spectrum, compared to that with glutathione, was a deflection at 275 nm.

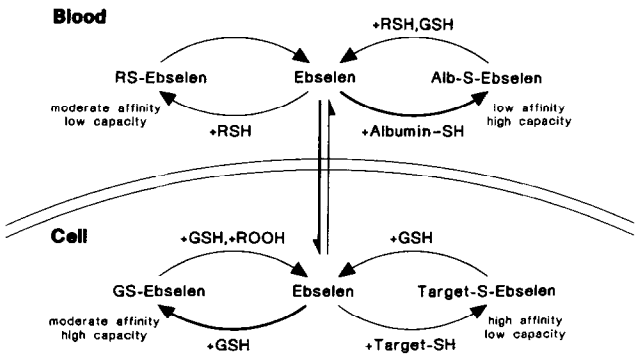
Whether or not this is related to changes in aromatic amino acids of the protein or in ebselen itself was not further investigated. From our kinetic data, in addition to the GSH-sensitive binding, one can observe a pool of ^{14}C-ebselen that exchanges slowly and is independent of added GSH. There may even be a third portion that is tightly bound and not exchangeable, which could be identical to the guanidinium cyanate-sensitive binding reported by Wagner *et al.* [10].

Our results show that a spectral interaction of ebselen with intact human PMN is qualitatively different from that with albumin. All of it is thiol-dependent because pretreatment with NEM abolished the complete difference spectrum. Depleting the cells from GSH still allows the formation of a spectrum that has a red-shifted isosbestic point compared to albumin. It is remarkable that human platelets exhibit a different spectral interaction.

It was the main objective of this work to explain the distribution of a given dose of ebselen within the body. In the crucial experiment with a therapeutically effective dose in whole blood, we observed binding to albumin as well as to PMN. Although the full binding capacity of albumin and

the other extracellular proteins was not reached, ebselen was present in PMNs in sufficient concentrations to exert the inhibitory effects described in the literature. In whole blood, more than 10^{-4} M concentrations of total thiol compounds were present, allowing a dynamic equilibrium of ebselen with all thiol groups in the system. The *in vitro* experiment with ^{14}C-labeled ebselen bound to albumin, and its exchange through a dialysis membrane into PMN in the presence of GSH, supports this assumption. If albumin-bound ebselen is in direct contact with PMN the label is transferred to PMN-proteins, which is in agreement with the finding of Wagner *et al.* [10] with hepatocytes. They suggested an equilibrium involving thiol groups of plasma membrane-bound proteins facilitating the kinetics of this process.

Thus, the apparently complex kinetic behavior of ebselen can be fully understood by assuming fast equilibria between all thiol-containing constituents of the system. The amount of drug present in one pool is dependent on the capacity of the pool and its affinity to ebselen. The latter, certainly, is a function of the nucleophilic character of a thiol and its stereochemistry. Hence, target proteins for ebselen may possess more acidic thiol groups and/or environments that accommodate the ebselen molecule in an appropriate crevice of the protein with additional binding forces (e.g. hydrophobic pockets and hydrogen bonding between the benzanilide and peptide bonds) for the ebselen molecule. Such thiol regions in proteins are not unusual, and this may explain the plethora of targets that have been shown or proposed for ebselen. The scheme below summarizes the finding of how ebselen, in spite of its covalent binding to plasma proteins, can reach its target proteins.



References

1. Sies H, Ebselen, A selenoorganic compound as glutathione peroxidase mimic. *Free Radical Biol Med* **14:** 313–323, 1993.
2. Schewe T, Molecular actions of ebselen—an antiinflammatory antioxidant. *Gen Pharmac* **26**(6): 1153–1169, 1995.
3. Parnham MJ, Leyck S, Graf E, Dowling EJ and Blake DR, The pharmacology of ebselen. *Agents Actions* **32:** 4–9, 1991.
4. Dimmeler S,Brüne B and Ullrich V, Ebselen prevents inositol (1,4,5)-trisphosphate binding to its receptor. *Biochem Pharmacol* **42:** 1151–1153, 1991.
5. Hattori R, Inoue R, Sase K, Eizawa H, Kosuga K, Aoyama T, Masayasu H, Kawai C, Sasayama S and Yui Y, Preferential inhibition of inducible nitric oxide synthase by ebselen. *Eur J Pharmacol* **267:** R1–R2, 1994.
6. Kuhl P, Borbe HO, Römer A, Fischer H and Parnham MJ, Ebselen reduces the formation of LTB_4 in human and porcine leukocytes by isomerisation to its 5S, 12R-6-transisomer. *Prostaglandins* **31:** 1029–1048, 1986.
7. Oshita M, Takei Y, Kawano S, Fusamoto H and Kamada T, Protective effect of ebselen on constrictive hepatic vasculature: Prevention of alcohol-induced effects on portal pressure in perfused livers. *J Pharmacol Exp Ther* **271:** 20–24, 1994.
8. Kamigata N, Takata M, Matsuyama H and Kobayashi M, Novel ring opening reaction of 2-aryl-1,2-benzisoselenazol3(2H)-one with thiols. *Heterocycles* **24:** 3027–3030, 1986b.
9. Nomura H, Hakusui H and Takegoshi T, Binding of ebselen to plasma protein. In: Wendel, A (ed.): *Selenium in Biology and Medicine*, Springer Verlag Heidelberg, 189–193, 1989.
10. Wagner G, Schuch G, Akerboom TPM and Sies H, Transport of ebselen in plasma and its transfer to binding sites in the hepatocyte. *Biochem Pharmacol* **48:** 1137–1144, 1994.
11. Renson M and Piette J-L, Etude des propriétés du chlorure d'o-chloroséléobenzoyle application à la synthèse d'o-alcoylsélénoacylophénones. *Bull Soc Chim Belg* **13:** 507–517, 1964.
12. Brüne B and Ullrich V, Different calcium pools in human platelets and their role in thromboxane A_2 formation. *J Biol Chem* **266**(29): 19232–19237, 1991.
13. Brüne B and Ullrich V, Inhibition of platelet aggregation by carbon monoxide is mediated by activation of guanylate cyclase. *Mol Pharmacol* **32:** 497–504, 1987.
14. Hatzelmann A and Ullrich V, Regulation of 5-lipoxygenase activity by the glutathione status in human polymorphonuclear leukocytes. *Eur J Biochem* **169:** 175–184, 1987.
15. Haenen GRMM, De Rooij BM, Veremeulen NPE and Bast A, Mechanism of the reaction of ebselen with endogenous thiols. *Mol Pharmacol* **37:** 412–422, 1989.